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QUARTERLY

Actinomycin D specifically inhibits the interaction between transcription factor Sp1 and its binding site*°

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The mode of action of many anticancer drugs involves DNA interactions. We here examine the ability of actinomycin D to alter the specific binding of transcription factors Sp1 and NFκB to their DNA sequences. Employing an electrophoretic mobility shift assay, it is shown that actinomycin D inhibits complex formation between nuclear proteins present in the extracts from stimulated human umbilical vein endothelial cells and the Sp1-binding site. Actinomycin D is also able to induce disruption of preformed DNA-protein complexes, pointing to the importance of an equilibrium of three components: actinomycin D, protein and DNA for drug action. The effect of actinomycin D is sequence-specific, since no inhibition is observed for interaction of nuclear proteins with the NFκB binding site. The results support the view that DNA-binding drugs displaying high sequence-selectivity can exhibit distinct effects on the interaction between DNA and different DNA-binding proteins.

It is well known that regulation of gene expression, both tissue-specific and in response to external stimuli, is mediated at the transcriptional level by formation of complexes between transcription factors and specific nucleotide sequences in promoter and enhancer regions of the genes. These contacts between DNA and regulatory proteins may be affected by anticancer drugs and related ligands, leading in turn to alteration of the transcription [1]. It is particularly interesting to study the effects of ligands on DNA-protein interaction if the DNA sequences recognized by a protein

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Abbreviations: HUVEC, human umbilical vein endothelial cells; Sp1, stimulatory protein 1; NFκB, nuclear factor κB; LPS, lipopolysaccharides; EMSA, electrophoretic mobility shift assay.

are well defined. These effects are followed by means of footprinting experiments and electrophoretic mobility shift assays [2–7].

In our experiments we studied the effect of actinomycin D on Sp1 and NFκB interactions with their target sequences. Actinomycin D binds *via* intercalation of the planar chromophore, preferably at the GpC sequence, with the drug pentapeptide rings lying in the minor groove of DNA helix. As the Sp1-binding site contains GpC sequences while the NFκB-binding site contains clustered GpGpG but no GpC (Scheme 1), they may be considered as sequences of choice making it possible to distinguish between specific and non-specific ligand effects. As a control ligand netropsin, the A·T specific minor groove binder, was used. NFκB was derived from HIV-1 enhancer [8]. Sequences containing Sp1-binding sites came from the promoter region of the gene for α_v subunit of human vitronectin receptor [9].

Sp1 5′-AATTCCCCGCCCCCGCCCCC-3′

NFκB 5′-AATTTCCGGGACTTTCCCACC-3′

Scheme 1. Oligonucleotides used in this study.

Binding sites for Sp1 and NFκB are underlined.

Crude nuclear extracts from human umbilical vein endothelial cells (HUVEC) stimulated with lipopolysaccharide (LPS) were used as a source of transcription factors.

MATERIALS AND METHODS

DNA-binding drugs. Stock solutions of actinomycin D and netropsin (0.2 mM) were stored at −20°C in the dark and diluted immediately before use.

Oligonucleotides. The oligonucleotides were synthesized and purified in the laboratory of Professor W.T. Markiewicz (Polish Academy of Sciences, Poznań). The double-stranded oligonucleotides were then labelled by filling in the overhangs with Klenow enzyme in the presence of [α-^{32}P]dATP, Amersham (England), and subsequently purified by 7% polyacrylamide gel electrophoresis in 0.5 × Tris/borate/EDTA.

Preparation of nuclear factors from human umbilical vein endothelial cells stimulated with lipopolysaccharide. Endothelial cells were isolated from human umbilical vein and cultured as described by Jaffe *et al.* [10]. Cells from the second passage were used for extract preparation. Nuclear extracts from confluent human endothelial cell cultures treated for 2 h with 100 ng/ml of LPS, Sigma, were prepared essentially as described by Dignam *et al.* [11], except that modified buffer C was used [12].

Electrophoretic mobility shift assays (EMSA). Binding reactions were performed in the binding buffer (20 mM Hepes/KOH, pH 7.9, 50 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol) in the presence of a non-specific competitor poly (dI:dC)·poly (dI:dC), Pharmacia. Crude nuclear extracts, 5–10 μg, from LPS stimulated HUVEC were incubated with 0.2 ng of ^{32}P-labelled oligonucleotide for 15 min at room temperature in a total volume of 20 μl. Where indicated, 20 ng of unlabelled oligonucleotides, either homologous or non-homologous, were added to the reaction mixture. DNA-binding drugs were added either prior to the addition of nuclear extracts and were incubated with the radioactive probe for 15 min or, as in displacement experiments, after the protein–DNA complex formation was completed. Protein–DNA complexes were resolved by 7% polyacrylamide gel electrophoresis in 0.5 × Tris/borate/EDTA at a constant voltage

(150 V for 1.5 h). Gels were vacuum-dried and autoradiographed with intensifying screens at -20°C for 2 to 18 h [13].

RESULTS

Actinomycin D-mediated inhibition of the interaction between nuclear proteins and the dimer of the Sp1-binding site

In the electrophoretic mobility shift assay (EMSA) one major retarded band was observed when HUVEC stimulated with LPS were incubated with ^{32}P-labelled double-stranded oligonucleotide containing two Sp1-binding sites (Fig. 1, lane 1). It is likely that transcription factor Sp1 present in nuclear extracts is involved in interaction with the labelled oligonucleotide since purified recombinant Sp1 also binds that element causing migration at the same position (not shown). The specificity of the protein-DNA interaction was confirmed by complete competition of the binding with a 100-fold molar excess of unlabelled homologous oligonucleotide and the lack of competition with a 100-fold molar excess of non-homologous double-stranded NFκB oligonucleotide containing no GpC sequences (Fig. 1, lanes 2 and 3, respectively).

In the experiments conducted in the presence of different concentrations of actinomycin D (0.1-5 μM) suppression of a retarded band was observed (Fig. 1, lanes 4-6). Actinomycin D at a concentration of 5 μM almost completely abolished complex formation. By contrast, netropsin which binds preferentially to the A+T sequences, was unable to suppress the binding of nuclear proteins to the dimer of the Sp1 site even at a concentration as high as 10 μM (Fig. 1, lanes 8 and 9).

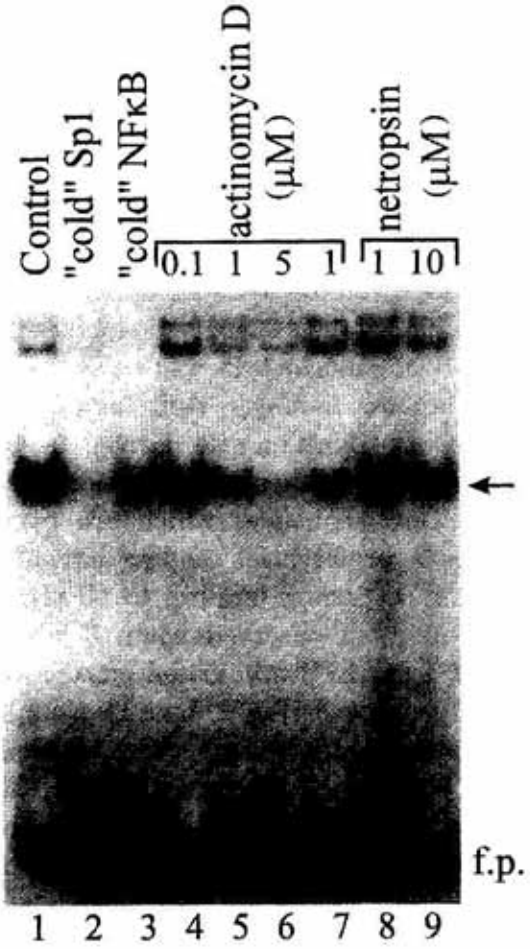

Figure 1. Effect of actinomycin D and netropsin on molecular interaction between nuclear factors and the dimer of the Sp1-binding site.

Nuclear factors were isolated from human umbilical vein endothelial cells induced for 2 h with 100 ng/ml LPS. A ^{32}P-labelled oligonucleotide containing two Sp1-binding sites was used in the electrophoretic mobility shift assay. Control binding reaction performed in the absence of DNA-binding drugs (lane 1). Competition was performed with a 100-fold molar excess of unlabelled oligonucleotide either specific (lane 2) or nonspecific containing a NFκB motif (lane 3). The effects of different concentrations of either actinomycin D or netropsin are shown in the lanes 4-6 and 8-9, respectively. The labelled oligonucleotide was first incubated with the indicated amount of drug for 15 min and then nuclear extracts were added and incubated for another 15 min. Displacement of protein from the pre-formed DNA-protein complex by actinomycin D is shown in lane 7. First, nuclear proteins were incubated with the DNA probe and then actinomycin D was added at a concentration of 1 μM and incubation was prolonged for another 15 min. The protein-DNA complex is indicated by arrow, free probe by f.p.

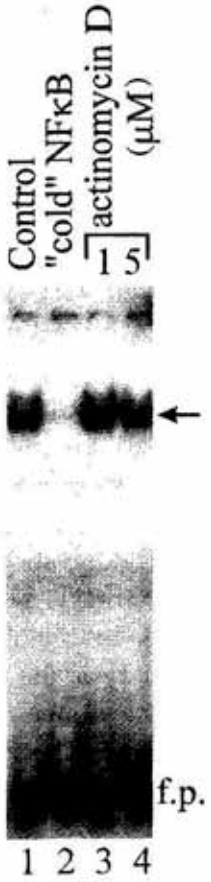

Figure 2. Actinomycin D does not interfere with DNA–protein complex formation when there is no GpC present in the binding site.

In control experiment a ^{32}P-labelled oligonucleotide containing the NFκB motif of HIV-1 enhancer was incubated for 15 min with nuclear extracts from LPS stimulated human umbilical vein endothelial cells either without (lane 1) or with a homologous competitor (lane 2). The same reactions were performed in the presence of actinomycin D at concentrations of 1 μM and 5 μM (lanes 3 and 4). The oligonucleotide was first incubated with actinomycin D for 15 min and then nuclear extracts were added. Arrow, protein–DNA complex retarded in the bandshift experiments; f.p., free probe.

Most of the results were confirmed in four independent experiments in which different preparations of nuclear proteins from stimulated HUVEC were used. All of them pointed to actinomycin D-mediated inhibition of the interaction between transcription factors and Sp1-binding sites.

Actinomycin D can target the Sp1–DNA complex

In the experiments presented above the labelled oligonucleotide was first incubated with actinomycin D for 15 min to allow full binding and then nuclear extracts were added to form DNA–protein complexes for another 15 min. About 50% inhibition was obtained when 1 μM actinomycin D was used (Fig. 1, lane 5). To determine whether Sp1- binding to the specific oligonucleotide could be targeted by actinomycin D, preformed DNA–protein complexes were incubated with 1 μM actinomycin D. The same level of inhibition was observed (Fig. 1, lane 7), suggesting that the protein complexed with the oligonucleotide can be substituted by actinomycin D to a determined level at certain concentrations of the drug. The percentage of inhibition was estimated by scanning the autoradiograms (not shown).

Actinomycin D inhibits specifically the molecular interaction between transcription factor Sp1 and its binding site

In order to determine whether actinomycin D at a given concentration specifically inhibits the molecular interaction between nuclear proteins and the dimer of the Sp1-binding site, ^{32}P-labelled double-stranded NFκB oligonucleotide was used in the electrophoretic mobility shift assay (Fig. 2). The NFκB binding site derived from HIV-1 enhancer was chosen since it does not contain GpC sequences which are considered to be a preferential binding site of actinomycin D. Strong binding to NFκB oligonucleotide was observed when a nuclear extract from LPS stimulated cultures of HUVEC was used (Fig. 2, lane 1). Complex formation was specific since the labelled probe can be competed with a 100-fold excess of unlabelled oligonucleotide (Fig. 2, lane 2). The presence of actinomycin D in the incu-

bation mixture did not interfere with complex formation between nuclear proteins and the NFκB-binding site at either 1 μM or 5 μM concentration of the drug (Fig. 2, lanes 3 and 4).

DISCUSSION

Effects of DNA interacting drugs and carcinogens on regulatory transcription factors have attracted much attention, particularly since the specific sequences that these proteins recognize on DNA have been identified ([6, 7, 14–18] and references therein). It was shown that Sp1–DNA complex formation is inhibited by mithramycin and chromomycin, two antibiotics exhibiting a preference for d(GC) rich sequences while distamycin A, a d(A·T) specific ligand, and its analogs were ineffective [7]. Actinomycin D also exhibits d(GC) affinity but, in contrast to mithramycin or chromomycin, it forms a well defined intercalative complex with double-stranded DNA [17]. Our study shows that, consistently with its DNA specificity, actinomycin D inhibits association of nuclear proteins to Sp1-binding sites. Radioactivity measurements indicate that the complex formation is decreased to 50% at the drug concentration of 1 μM and almost abolished at 5 μM. This result may be compared with the inhibitory effect of actinomycin D on RNA synthesis *in vitro*. Although transcription factor association with DNA and RNA synthesis *in vitro* are obviously two different systems, the latter may be considered a reference system which is less dependent on the drug sequence specificity. DNA dependent RNA synthesis under conditions used in our laboratory reduced by half at 0.4 μM concentration of the drug [18]. In mammary adenocarcinoma cells, actinomycin D at a concentration of 4 μM abolished the stimulatory effects of phorbol ester on the matrix metalloproteinase and the tissue inhibitor of matrix metalloproteinase [19]. These results indicate that the concentration of actinomycin D used in our studies was sufficiently low to allow us to claim that actinomycin D could inhibit transcription directed by promoters containing the Sp1-binding site also by decreasing the efficiency of complex formation between the ubiquitously occurring transcription factor Sp1 and its binding site. The effect of actinomycin D on Sp1-binding depends on the equilibrium of three components: drug, protein and DNA as a similar inhibition is observed when the drug is added to the preformed DNA–Sp1 complexes.

The effect of actinomycin D is sequence specific. Even at higher concentration it does not affect association of NFκB to its canonical sequence devoid of GpC (Scheme 1). Its effect depends also on protein–DNA contacts as this antibiotic does not cause an appreciable alteration in binding of several transcription factors with their binding sites, some of them containing GpC sequences [16]. The importance of drug sequence specificity for the inhibition of Sp1-binding is strengthened by inability of netropsin, a d(A·T) specific antibiotic, to prevent complex formation. We show here that netropsin does not appreciably inhibit Sp1-binding even at a 10 μM concentration (Fig. 1). That concentration is otherwise high enough to inhibit RNA synthesis *in vitro* by 50% [20].

As mentioned above, actinomycin- and netropsin–DNA complexes differ in their steric properties. The actinomycin chromophore is intercalated and its peptide lactone rings interact in the minor groove. To evaluate the possibility that any intercalating drug may effectively compete with Sp1 for its binding site we assayed several acridine derivatives forming intercalative complexes with DNA (not shown). Inhibition up to 10–20% was found at drug concentrations as high as 10–20 μM. In the case of triacridine, one of the strongest bis-

intercalative derivatives, Sp1-binding was decreased to 60% at the drug concentration of 50 μM which is otherwise over 10-fold higher than that needed to decrease RNA synthesis *in vitro* to 50% [21]. Therefore it can be concluded that sequence specificity of actinomycin D plays a crucial role in this drug mediated inhibition of Sp1-binding to DNA.

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